SURFACE MODIFICATION FOR BIOCOMPATIBILITY

Final Report

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Project Overview

This research addressed the interactions of materials with tissue of the central nervous system (CNS), with a long-term goal of rationally designing microelectrode surfaces to promote specific tissue interactions. To this end, the primary objectives of the project were to develop an *in vitro* cell culture model and to use *in vitro* cultures to assess the effect of surface modifications that control the interaction of neurons, astrocytes, and/or microglia in embryonic to adult stages of development. Much of the resultant data has been summarized in the Quarterly Reports.

In this final report we have summarized the primary results and conclusions of the project, defined the limitations we encountered and provided our recommendations for future study. Our research focused on and addressed the following objectives:

1. Develop a cell culture or other suitable model of mammalian cortex and investigate the growth and adhesion of neurons, glia, microglia, and other cells present in the central nervous system on substrates coated with the selected surfaces.
2. Prepare surfaces modified by SAMs or SAMs modified with biological molecules.
3. Characterize surfaces before and after cell culture using surface analytical techniques including X-ray electron spectroscopy (XPS), ellipsometry, contact angle measurements, and biological assay.
4. Screen self-assembled (SAM) and SAMs – biological surfaces using serum-free *in vitro* cell culture (embryonic rat cortical cells will be cultured on different SAMs to find the SAM and SAM + macromolecular surfaces with the ability to support neuronal survival and axonal outgrowth, or those surfaces which do not support neuronal growth but enhance the survival of cortical glia).

5. Attach selected organic surfaces to the electrode materials: SiO_2, Si_3N_4, teflon, and iridium.
6. Cooperate with other investigators in the Neural Prothesis Program by coating microelectrodes (estimated 50 over the contract period with the most promising materials for *in vivo* evaluation as directed by the NINDS Project Officer.

Major Accomplishments

1. The major accomplishment of this project was the establishment of a defined serum-free *in vitro* system for testing surface coatings and factors introduced in the media. We also showed this defined system could be used to culture cortical neurons, astrocytes, and microglia. Finally, but most importantly, we have shown that this system is valid for the culture of embryonic precursor cells and to follow their development through to adult cells, as well as adult neuronal culture. We believe this is the first time adult culture has been achieved with cortical cells. The program now has access to a culture system for testing new surface coatings that allow exploration of most of the primary variables found in stable *in vivo* systems.
2. Surface coatings of self-assembled monolayers were applied to SiO_2 (from glass or Si) and the stability was found to be poor but improved in the presence of protein. However, on another project we found that the glass surface can be stabilized with Ti and Zr doping of the SiO_2 surface. We had previously shown that these SAMs could be patterned by UV lithography (Hickman et al., 1994). We confirmed this is also possible with cortical cells. We did not have the time to extend this to teflon or Si_3N_4 but other researchers have shown these surfaces can be modified with SAMs and we expect the culture results to be similar. Iridium was not tested.

3. We have developed a new diagnostic tool for looking at proteins on surfaces in a defined system. We have examined by XPS surface analysis the amount of biological material deposited from the media in the absence of cells and found it to be minimal. However, the material deposited by the cells during culture appears to correlate directly to cell survival and morphology. This is another advantage of our new defined culture system.
4. Certain biological macromolecules were immobilized and tested for cell culture. The coupling chemistry was eventually worked out but the culture results were inconclusive. This approach was not vigorously pursued due to the large variation in cell response to the SAMs alone.
5. No actual mechanical stabilization experiments were done, but our results indicate both positive and negative surfaces can be created and this combined with low resolution patterns could provide differently modified electrodes. These now just need to be tested in an *in vivo* environment. We coated 42 microelectrodes with a positive and negative coating and sent them to our collaborators, but the project ended before testing.

Synopsis of Results

Surface Coatings

The list of SAMs that have been examined for their effect on cell culture survival and axonal outgrowth are shown in Table 1. The average water advancing contact angles are given for each monolayer in descending order starting with the most hydrophobic surface. The contact angle measurement provides a measure of the surface energy of the surface and combined with XPS is a reliable measure of reproducibility of surface modification in our defined system. For example, in Table 1, OTS has the highest average contact angle of 103° and hence, the lowest surface energy. Representative XPS signals of SAMs on SiO_2 can be found in Report 1.

Table 1

SAMs examined in cell culture, their acronyms, chemical names and contact angles

Abbreviation	Chemical	Contact angle, θ_{adv} (degrees)
OTS	octadecyltrichlorosilane	103
NDEC	N-decyltrichlorosilane	98
13F	(tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-dimethylchlorosilane	90
TCMD	(10-Carbomethoxydecyl) dimethylchlorosilane	67
PPDM	(3-phenylpropyl)dimethylochlorosilane	64
CP	3-cyanopropyldimethlchlorosilane	63
TP	triphenylchlorosilane	60
PEDA	(aminoethylaminoomethyl)phenethyltrimethoxysilane	56
MTS	3-mercaptopropyltrimethoxysilane	54
MTS-OX	oxidized 3-mercaptopropyl-trimethoxysilane	46
NBUT	n-butyldimethylchlorosilane	44
APTS	aminopropyltrimethoxysilane	42
DETA	trimethoxysilylpropyldiethylenetriamine	37
EDA	N-(2-aminoethyl)-3-aminopropyltrimethoxysilane	35
MAP	N-methylaminopropyltrimethoxysilane	34
D-MAP	(N,N dimethyl-3-aminopropyl)trimethoxysilane	31
PEG-350	triethoxysilyl polyethyleneglycol (mw = 350)	30
UAD	11- undecanoic acid dimethylsiloxy	27

Stability of SAMs

For use *in vivo*, it is important to assess the stability of the SAMs under physiological conditions. Initially, eight (8) SAMs were tested on glass coverslips in Dulbecco's phosphate buffered saline (D-PBS) at 37°C. With the possible exception of the MTS SAM, there was evidence of a decrease in molecular coverage after two weeks. This can be attributed to hydrolysis, as has been reported by other workers.

In a second study, a buffered solution of protein using the concentration found in our *in vitro* serum-free cultures (bovine serum albumin, BSA, 0.001%) was investigated. For the highly fluorinated 13FCl monolayer, the XPS data supported a hypothesis that the attenuation of the F signal over time was caused by a rapidly deposited protein overlayer that adsorbs when BSA is present in solution. The presence of an adsorbed adlayer has been theorized to make some silanes relatively more stable. We hypothesize that the protein layer inhibits hydrolysis of SAM molecules from SiO_2. After 4-8 weeks, however, we observed a further decrease in the F signal and an increase of ions (Ti, Na) that are indicative of glass dissolving. In further experiments, the stability of trichloro- and monochloro-terminated 13F silane monolayers was tested on two different substrates, glass coverslips and silicon wafers in a higher concentration of BSA (0.1%). The trichloro 13F monolayer (on SiO_2) was found to be more stable than the monochloro 13F for short periods. This work is shown in detail in Reports 2, 3, 4, and 5.

Taken together, these studies indicate that proteins adsorbing to the surface of implanted electrodes will stabilize the SAM for an initial period. The reaction mechanism of the SAM with the surface is also a key element. SAMs with multiple attachment points (trichloro vs. monochloro) are more stable. The glass is unstable in the presence of PBS but we have results form another project to show we can stabilize the glass and then resultant monolayers. In this work, we found that doping the SiO_2 with Ti or Zr extended the lifetime of the SAMs on SiO_2 to over 90 days. Finally, since the main goal for the SAM is to be a template to initiate the incorporation of an

implant it may be enough for it to have short term stability.

XPS of Cell Culture Plates

The amount of material deposited on the various surfaces by cells in culture was measured by XPS. XPS peak areas for DETA with and without cells, indicates that the deposited material is from the cells and not from the surrounding serum-free medium (MEM/N3). This is extremely important for determining the response of cells in a defined *in vitro* system. In addition, in the presence of cells a protein layer is rapidly established and then levels off over time. There appears to be a relationship between cell morphology and attachment and the amount of protein deposited on a substrate, regardless of substrate type. Modeling of the normalized C 1s peak areas for DETA and PDL substrates which facilitate survival and attachment yield protein layer thickness of 60-70Å for both surfaces after 10 days in this medium. Substrates with thicker protein layers are generally less able to support survival and attachment of cells.

The examination of the deposited material after culture has proved to be very informative in characterizing the cells' response to surfaces and correlates with morphology. We have shown that in healthy cell cultures, in our model system, that the deposited material increases over a one to three day period and then stabilizes. This work is summarized in Reports 3 and 9.

Cell Culture Results

Embryonic cortical neurons from rat cortex (E12, 13, 14, 16, 18, 19, 22, and neonates) were cultured according to the methods of Schaffner et al. 1995, for hippocampal cells. (Report 1).

Astrocyte cultures were produced according to methods compiled by the laboratories of Schaffner and Barker at NINDS and described in Report 3.

Microglia cultures were prepared from 2 day old rat neonates according to the procedures developed by Dr. Carol Colton at Georgetown University for neonatal mouse or hamster microglial cultures (see Report # 5).

Phenotype Determination, Neurons, Astrocytes, Growth Factors

One of our primary goals was to determine if modified surfaces affect the adhesion, survival and differentiation of cells *in vitro*. In our most striking example, we found that DETA facilitated attachment and survival of cells, as compared to a 13F substrate. Other surfaces showed varying ability to support cell culture in our system. These results are discussed extensively in Reports 1-9. We also investigated the effects of SAMs on cell phenotype expression of rat cerebral cortex in defined system. Cell lineage studies have shown that there are two major types of neurons in the rat cerebral cortex, pyramidal and non-pyramidal cells (Price and Thurlow, 1988; Parnavelas et al., 1992). Pyramidal neurons contain the excitatory transmitter glutamate whereas most non-pyramidal neurons use the inhibitory neurotransmitter GABA (Jones and Hendy, 1986; Emson and Lindvall, 1989). By using antibodies against glutamate and GABA we are able to identify these two types of neurons. There are also three distinct types of glial cells: astrocytes, oligodendrocytes and microglia (Grove et al., 1993). Using specific cell markers (GFAP, OX42, O4) we can detect each of those types of glial cells. All the neurons and glial cells that comprise the adult cerebral cortex are derived from the embryonic precursors. Cell phenotype choice (whether to become neurons or glia) and neurotransmitter choice (whether to become glutamatergic or GABAergic) are a crucial step in cortical development and the factors that control these choices and the timely differentiation of the precursor cells into differentiated cell types are not well known. It is believed that both the cell environment and the intrinsic developmental program are important in regulating cell lineage (Williams and Price, 1995). In our studies, the expression of markers for cortical cell phenotype were examined in our *in vitro* system on silica substrates modified with artificial surfaces (see Report # 9). Double-immunofluorescence staining for glutamate and GABA was carried out in sister cultures (Report 9). Results showed that in cultures grown on PDL, glutamatergic neurons represent approximately 30-40% of total cells, GABAergic neurons represent approximately 50-60% of total cells,

and the ratio of glutamatergic to GABAergic cells was 0.6 : 1. This is consistent with the findings of immunocytochemical studies *in vivo* (Conti *et al.*, 1987; 1989). In cultures on DETA, this ratio was 0.60 : 1. In contrast, on 13F and OTS, about 20 - 30% of the cells were glutamatergic, 50-60% expressed glutamate decarboxylase (GAD) and the ratio of glutamatergic to GABAergic cells was 0.45 : 1. This suggests that a hydrophilic surface (DETA), similar to poly-D-lysine, supports both glutamatergic and GABAergic transmitter phenotypes, while hydrophobic surfaces (13F and OTS) decrease the number of glutamatergic cells more than GABAergic cells. This experiment indicates we can use surface chemistry to select for cell phenotype.

Another striking finding was from our studies on the effects of a combination of two factors (substratum and growth factor) on cell survival and differentiation. We investigated the effects of basic fibroblast growth factor (bFGF) on cell survival and process extension or astrocytes taken from new born rat cerebral cortex and grown on artificial surfaces (Report 9). The leading edge extension is characterized by the leading edge output, the branch nodes and the number of arbors. Results showed a striking trophic action of bFGF on astrocytes grown on poor surfaces (13F and OTS), but not on the cells on PDL and DETA. These findings strongly suggest that bFGF exerts some ameliorating effect at the cell-surface interface on astrocytes grown only on sub-par surfaces. This has also been demonstrated with neurons.

In another set of experiments, we investigated the effects of bFGF, brain derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF) on survival of neurons and neurite outgrowth on artificial surfaces. The cortical cells were grown on PDL, DETA, 13F, and OTS. Cell survival was assessed by a comparison of the number of cells present at a given point relative to the number of cells surviving at the time of culture initiation. The extent of neurite outgrowth was characterized in terms of total process output, the mean arbor output, the number of arbors and cell body area. Results showed that hydrophilic surface (DETA), similar to PDL, supported the growth of primary cortical neurons, whereas poor attachment and

consequent poor cell survival and the extent of neurite growth occurred in cultures grown on hydrophobic surfaces (13F and OTS). However, the addition of bFGF (20 ng/mL), BDNF (100 ng/mL), and CNTF (20 ng/mL) increased the number of cells surviving and the extent of neurite growth. Double immunofluorescence staining with a mixture of anti-glutamate (Incstar) and anti-GAD (courtesy of Dr. Kopin) in sister cultures showed that the growth factors enhanced cell survival and neurite outgrowth for both glutamatergic and GABAergic neurons.

Microglia

We have preliminary results indicating that the substrate can play a key role in microglial attachment, activation and response (Report 6 and 7). Microglia plated on PDL controls and DETA have improved attachment and survival as compared to cells plated on the 13F monolayer. The cultured microglia project thin processes and resemble ramified microglia. Microglial cultures of rat have proved to be difficult which decreased the amount of usable data. Our results may given some clues as to why teflon is an acceptable coating for implanted CNS materials.

Covalent Attachment of Biological Macromolecules

SAMs are important in the preparation of a reproducible, functionalized surface. SAMs can also be used to covalently attach biological macromolecules to the surface. The various SAMs shown in Table 1 provide a number of different functional groups with which to do further chemistry. The attachment of biological macromolecules to SAMs is done with crosslinkers such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), phenyldiisothiocyanate, N-succinimidyl 4-maleimidobutyrate (GMBS), or glutaraldehyde.

In addition to apo-transferrin (APO), bovine serum albumin (BSA) was also covalently attached to an APTS monolayer and tested in cell culture with E18 rat cortical neurons. While the outcomes are preliminary and subtle, both adsorbed and covalently linked BSA and covalently linked APO show

differences in survival, process outgrowth and cell population compared to controls as early as Day 4 (Reports 7 and 8).

Adult Culture Results

We have successfully cultured adult rat cortical cells in a defined, serum-free culture (Reports 7 and 9) with bFGF. Dissociated cells express the neuronal markers, microtubule associated protein (MAP-2). There are very few adult *in vitro* culture systems developed for mammalian cells to study injury in the adult CNS, with the exception of photoreceptors. Many researchers have utilized embryonic systems to analyze components of *in vivo* adult CNS, but until this study (and the studies of Brewer, 1997 with hippocampus) there has been little opportunity to utilize an adult *in vitro* culture system that uses defined medium and substrates for the study of cortical neuronal regeneration or for developing brain implants for adult CNS. This well defined *in vitro* system could be utilized to assess the environmental effects of molecules that may be limiting the regeneration of CNS neurons *in vivo.*, which are only partially understood by studies with embryonic cells in *in vitro* cultures (Fawcett, 1995; Johnson, 1995), and more uniquely, the genetic or inherent characteristics of the mature adult cortical neuron without *in vivo* environmental effectors. This *in vitro* system gives the opportunity to develop and to analyze not only adult cortical neuron response to injury but also could allow us to assess the effects of biomolecules, *in vivo* inhibitory components or tissues, allow the development or analysis of drugs that will play a direct role in the development of *in vivo* implants, and substrate modifications.

Cortical Precursor Cells

Results showed that at day 1, a few cells survived on SAM surfaces. Cell survival was similar on all three substrates, PDL, DETA, and OTS (Report 11). However, at Day 3, clusters of undifferentiated cells labeled by BrdU (not shown) were present on PDL, but not on DETA and OTS (Report 11). These

results indicate that neural precursor cells dissociated from rat telencephalic neuroepithelium can be expanded by basic fibroblast growth factor on PDL, but not on DETA and OTS.

Collaborations

We established a collaboration with Dr. Agnew at the Huntington Medical Research Institute early in this contract. We successfully modified the poly-silicate sheath that hold a microelectrode series in place along the spinal cord during implantation. This investigative interaction addressed the glial scar buildup and adhesions within the sheath which eventually displace the electrodes laterally (with adhesion) and vertically (due to buildup of tissue).

This fall, we supplied some uncoated electrodes to Dr. Cordell Gross at the University of Vermont. He performed some initial studies of *in vivo* implantation of these iridium electrodes into cortex with animal models in preparation for handling coated electrodes. We have recently sent iridium electrodes modified with the SAMs DETA and 13F to Dr. Agnew at the Huntington Institute and Dr. Cordell Gross for further *in vivo* studies and analysis. Dr. Agnew will be reporting back on his results in the near future.

Multivariate Analysis of Secondary Ion Mass Spectrometry Data from Adsorbed Proteins - University of Washington Collaboration.

We established a collaboration with Dr. Buddy Ratner and his student Fred Wolf to supply samples for analysis with SIMS methods being developed at University of Washington. This could complement our existing surface analysis techniques which can determine surface free energy, surface composition, and overlayer thickness. These methods can't identify individual macromolecules without labeling while SIMS may be able to identify specific proteins by their fragmentation pattern.

Several silicon chips coated with the SAMs OTS and DETA were exposed to fibronectin and sent for analysis. These samples were investigated with

secondary ion mass spectrometry (SIMS) and multivariate data analysis. A typical sample will produce a characteristic fragmentation pattern containing hundreds of different secondary ions, resulting in very large data sets. Therefore, multivariate techniques such as principal components regression (PCR) and partial least-squares regression (PLS) were employed to aid the analysis of the TOF-SIMS data. These regression techniques help find correlations between the independent variables (e.g. the observed fragmentation pattern from the TOF-SIMS instrument) and user-defined dependent variables (e.g. protein type or conformation). A successful multivariate model may be able to identify the type of protein on the surface or help characterize its structural conformation or orientation relative to the surface. While preliminary, this work has great promise for future studies using our defined system.

Portions of Statement of Work Not Addressed.

Comments regarding limitations to the full achievement of some portions of the statement of work are addressed point by point, numbered according to the original statement of work, (pg. 2 above).

Project Objective 2. Most of our effort was spent assessing the interactions between cells and surfaces (silanes on glass) and their time course in culture. The scope of the investigations with biological molecules covalently linked to silanes on glass was therefore limited. Our findings about biomolecules on surfaces should be considered preliminary results as the number of trials was small (N=1,2). On the other hand, more effort was spent establishing the chemistry and examining the integrity of the biomolecules. Biomolecules were not applied to silicon nitride, teflon, or iridium surfaces.

Project Objective 4. We did identify a number of surfaces, some positive and some negative, for cell adhesion and growth. We found some surfaces yielded populations enhanced for expression or certain neurotransmitters, possibly via selective adhesion or by triggering genetic cues. However, we did not find a surface treatment that unambiguously differentially supported

neurons and discouraged glia. There were only a few studies of cellular attachment and viability on biomolecular surfaces, due to time constraints. Mixed cultures of cells to be used for the assay of such a phenomena were not pursued for the same reason. Examinations of microglial cultures were difficult because we had trouble with the procedure. Substrate preferences for these cells were similar to those found for neurons and glia, but trials were very limited and only preliminary data was obtained. Experiments with postnatal cells were limited due to the success of the adult cultures.

Project Objective 5. (see 2).

Project Objective 6. Some electrodes were coated with a single silane, DETA or 13F, and given to Dr. Agnew and Dr. Cordell Gross for implantation and examination. Single electrodes were not differentially coated because this was deemed premature until some *in vivo* results were obtained.

Recommendations for Future Research and Development.

Recommendation 1.

Short Term *in vivo* Culture Study to Validate *in vitro* System.

We have established a defined *in vitro* culture system and evaluated a number of surface modifications and growth factors, with specific cell types. It is necessary to validate this system for *in vivo* relevance. The short term experiments outlined in section 5.4.3 of the revised proposal involving leukocyte activation in response to acutely implanted SAM modified surfaces would be a good evaluation combined with immunostaining for specific proteins and cell type deposited in response to the implant. Once the system is validated it can become one of the few standards for evaluating CNS implants outside the body and then be expanded to include more complicated assays.

Recommendation 2.

Long Term *in vivo* Testing of Modified Surfaces with SAMs.

The purpose of this contract was to develop strategies for improving biocompatability of stimulating devices implanted in the nervous system.

We established a chemically defined *in vitro* to screen the SAMs. We found that DETA promotes neural cell survival and neurite growth and is an excellent SAM substrate for cortical cell culture, while cells on 13F (or OTS) showed poorly adhesion and growth. It is necessary to test the results for modified materials implanted into the brain in long term (2-6 month) studies. Unlike Recommendation 1 the efficacy of the approach would be to determine by signal transduction methods, stimulating current parameters, and life time of the implant followed by histology. This should be done in collaboration with other members of the Neural Prothesis Workshop.

Anticipated Publications

1. Hickman, J.J., Coulombe, M.G., Ma, W., and Sathanoori, R.: Development of an *In vitro* System to Study Neuronal Development as Well as Regeneration of Axonal Processes of Explanted Adult Neurons.

2. Hickman, J.J., Coulombe, D.R. Jung, P., Ma, W., H. Schessler: Differential Response of Astrocytes and Neurons to Artificial Surfaces with and without bFGF and Implications for CNS Implants.

3. Hickman, J.J., Jung, D.R., and Coulombe, M.G.: Correlation of Cortical Neuronal Morphological analysis to Material Deposited on Culture Substrate by XPS.

4. Jung, D.R., Coulombe, M.G., Bateman, K.F., Sathanoori, R.S., Schaffner, A.E., Barker, J.L., Stenger, D.A., and Hickman, J.J.: XPS Analysis of Protein Layers Deposited by *in-vitro* Neuronal Cell Culture.

5. D. Kelly, T. Schneider, J.J. Hickman: Surface Stability of SAMs in a Biological Environment.

6. Ma, W., Coulombe, M., Hickman, J.J.: Selective Expression of GABAergic and Glutamatergic Neurons on Organosilane Surfaces.

We have also attached a copy of a recent paper from our group showing the state-of-the-art in patterning entitled: "Developmental Neurobiology Implications from Fabrication and Analysis of Hippocampal Neuronal Networks on Patterned Silane-Modified Surfaces." Please see attachment.

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Developmental Neurobiology Implications from Fabrication and Analysis of Hippocampal Neuronal Networks on Patterned Silane-Modified Surfaces

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Abstract: We have determined the parameters necessary to fabricate *reproducible* neuronal patterns which we are using to study fundamental issues in developmental neurobiology. A beam homogenizer, as well as other advances in surface preparation, has enabled the routine production of reproducible, high resolution (2-20 μm) organosilane patterns. The effects of surface preparation and beam dosage were monitored using X-ray photoelectron spectroscopy (XPS) and proof of patterning is provided by high resolution imaging XPS. We report the guidance of neuronal adhesion and neurite outgrowth and the creation of reproducibly defined circuits of embryonic (E18-19) rat hippocampal neurons using these patterned surfaces *in vitro*. We have achieved a >50% rate of pattern formation and at times the rate approaches 90%. We have used these patterns to address the issue of how geometric pattern cues might be used to affect cell-to-cell communication and to report on the synaptic development of the

hippocampal neurons using dual patch clamp electrophysiology. We monitored neurite outgrowth and the emergence of both spontaneous and evoked synaptic activity for both patterned and unpatterned (control) hippocampal cultures. The results indicate the intriguing possibility that geometry itself may be a modulating or trophic factor for cell development.

Introduction

We have determined parameters for fabricating reproducible neuronal patterns on organosilane surfaces for studying fundamental developmental questions in neurobiology. X-ray Photoelectron Spectroscopy (XPS) was used in both the high energy resolution and imaging modes as a diagnostic tool for the optimization of organosilane photolithographic patterns with high resolution features. The use of a beam homogenizer, an optimal surface preparation and laser dosage allowed for the routine production of high resolution surface patterns. The combination of the optimized patterned surfaces and defined cell culture conditions resulted in high fidelity neuronal circuits of embryonic rat hippocampal neurons *in vitro*. We show that synaptic development and neurite outgrowth on these neuronal patterns are geometry dependent, and raise the possibility that surface pattern geometry may be a modulating or trophic[1] factor for cell development.

In the work presented here, we impose order on circuits of hippocampal neurons *in vitro* in an attempt to mimic the intrinsic high degree of order of *in*

vivo cells in the hippocampus (the region of the brain thought to be responsible for learning and memory functions).[2,3] There are three distinguishable neuronal phenotypes, or subpopulations, of neurons in the mature hippocampus: pyramidal cells, granular cells, and interneurons. Different regions of the hippocampus (*e.g.*, CA1, CA2, and dentate gyrus) contain different proportions of these three subpopulations. Electrophysiological and immunohistochemical examination of mature hippocampal neurons *in situ* and in slice preparations has revealed that the pyramidal and granular cells are excitatory while the interneurons are inhibitory. Both types of signals harmonize in slice preparations to generate long-term changes in fast synaptic transmission, a phenomenon considered to be a cellular correlate of learning. Different pathways through this complex cytoarchitecture have been identified that relate to different cognitive functions.[4] Understanding how the distinct regions of the hippocampus develop and what factors influence the differentiation of pre-mitotic cells into specific cellular phenotypes are some of the challenging issues in developmental neurobiology.

Efforts to unravel the physiological cues underlying the development of neuronal and glial phenotypes and circuit formation *in vivo* have been complemented by rudimentary *in vitro* systems designed to assess the cellular and molecular mechanisms for these processes. Monolayers of embryonic neurons dissociated from the mammalian central nervous system (CNS) and maintained in culture retain cellular and molecular properties reminiscent of

those found *in vivo*. Dissociated primary cultures of CNS cells are thus often used for studies designed to elucidate various aspects of neuronal and glial differentiation, including morphogenesis,[3] membrane excitability,[4] cell-cell communication,[5] and signal transduction.[7] Some aspects of neuronal differentiation have been studied extensively because they are readily reproducible, however, the experimental results and outcomes may vary due to the complexity and the empirical rationale of the methodology.[3,6,8]

One of the difficulties in the study of neuronal circuit formation among differentiating neurons and glial cells with presently available protocols is that circuits tend to form in an apparently random manner. Greater experimental control would facilitate the study of the pre- and post-synaptic physiological signals occurring during various stages of circuit formation. Etched grooves,[10-12] conventional photoresist technology,[13] plasma modification,[14] Langmuir-Blodgett films,[15] metal evaporation,[16] and photolithography,[17-26] have been utilized to impose order in dissociated cultures by patterning surfaces to control the growth of neurons and other cells. Kleinfeld *et al.*[13] first reliably demonstrated that embryonic mouse spinal cells and perinatal rat cerebellar cells could be confined to grow *in vitro* on patterned organosilane surfaces prepared using conventional photoresist technology. These patterned cerebellar cells developed both electrical excitability and immunoreactivity for neuron specific enolase which is a marker specific for neurons. The use of deep UV photolithography, which allows for higher resolution patterns, was adopted by

subsequent investigators[17-27] as a more versatile adaptation of the Kleinfeld approach.[3] Others[17,19,23] demonstrated the use of deep UV photolithography of self-assembled monolayers (SAMs) to make alternating line patterns of various widths (line-space patterns) to guide the outgrowth of processes from neuroblastoma cells, explanted rat hippocampal cells, and human umbilical vein endothelial cells. Corey *et al.*[24] created grid patterns of hippocampal neurons in a low density culture. Healy *et al.*[25] demonstrated the ability to organize bone cells and mineralize tissue *in vitro* on lithographically patterned organosilane surfaces. Chen *et al.*[26] recently used micropatterned surfaces to switch human and bovine capillary endothelial cells from growth to apoptosis using localized geometric control of cell growth. Our strategy improves on prior work with neurons because we have shown that SAMs can organize high resolution neurite outgrowth. Reproducible high resolution surface templates should facilitate the study of the factors influencing the formation, maintenance, and modulation of these rudimentary neuronal circuits.

The methodology for using deep UV photolithography to pattern SAMs is relatively new,[17-27] and the critical variables for optimizing this technique are still under investigation. We have previously[23] characterized the mechanisms involved in the photoinitiation process, and we have investigated various combinations of SAMs in an attempt to improve neuronal viability. Only a few molecular combinations (*e.g.*, DETA/13F) were shown to be effective at creating the differential adhesion properties necessary for neuronal patterning. In a

recent study, Corey showed that optimized laser conditions produced the best fidelity to a DETA grid pattern, with approximately one third of the background regions free of connected cells and neurites.[24] However, considerable variation across the surface was noted (*i.e.*, maximal fidelity near the center region of the surface, decreasing with increasing distance from the center). They hypothesized that regional intensity differences in the laser beam profile could be responsible for the variability in pattern fidelity.

XPS is an excellent diagnostic tool for characterizing surfaces.[28,29] XPS can be used in the high energy-resolution mode to provide the elemental surface composition and to resolve the oxidation states of individual elements of the surface preparation which allows for the determination of the optimal laser dosage for patterning. XPS can also be used in the imaging mode to provide an element specific photoelectron image from an organosilane surface which has been patterned. Based upon the results, we introduced a beam homogenizer in our process to create uniformity in the energy density of the laser beam, and combined this with advances in surface preparation and optimized laser dosages to fabricate high resolution patterns. The optimized high resolution circuit patterns successfully guided the neuronal adhesion and neurite outgrowth of E18-19 hippocampal neurons in a defined serum-free medium.

Using patch clamp techniques,[30] we report preliminary results on how geometric cues might be used to affect neurite outgrowth and cell-to-cell communication. We monitored the emergence of both spontaneous and evoked

synaptic activity over time in culture for both patterned and unpatterned (control) hippocampal cultures. Our preliminary results presented here indicate that **geometry** itself can serve as a **modulating** or **trophic**[1] factor for cell development.

Materials and Methods

Materials for Film Deposition. Trimethoxysilylpropyldiethylenetriamine (DETA) was purchased from United Chemicals (Bristol, PA). 1H,1H', 2H, 2H'-perfluorodecyldimethylchlorosilane (15F) was purchased from PCR, Inc. (Gainesville, FL). Sure-seal toluene was purchased from Aldrich (Milwaukee, WI). The DETA, 15F, and sure-seal toluene were stored in a Labmaster 130 glove box (MBraun, Innovative Technologies, Inc.) under inert conditions. HPLC-grade toluene, HPLC-grade methanol, reagent-grade hydrochloric acid, and reagent-grade sulfuric acid all from Fisher (Fairlawn, NJ) were used as received.

Surface Preparation. Thomas Red Label Micro Cover Glasses 22 x 22 No. 1 (Thomas Scientific) were cleaned in a series of steps. Ceramic racks (Coors, Inc.) containing the coverslips were soaked in a solution of 50/50 methanol/hydrochloric acid (15 min. minimum); rinsed with deionized water (3 rinses); soaked in concentrated sulfuric acid (30 min. minimum); rinsed with deionized water (3 times); boiled in deionized water (30 min. minimum); rinsed with acetone (two rinses); and oven dried (15 min., 110°C).

The cleaned coverslips were then immersed in a 0.1% (v/v) DETA in toluene solution and heated to just below the boiling temperature (30 min.). The coverslips were rinsed with HPLC-grade toluene (two rinses), re-heated to just below boiling in toluene (30 min.), and then oven dried (2 hrs.).

After the DETA-derivatized coverslips were patterned via laser irradiation (see below), the 15F rederivatization or backfill was completed by immersing the rack containing the patterned coverslips in 0.5% (v/v) 15F in toluene solution (1 hr.). The coverslips were then rinsed with HPLC-grade toluene (3 times) and oven dried (15 min. minimum).

Photolithographic Mask Design. The photolithographic mask used in this work was designed to investigate how geometric cues might be used to control cell growth and affect cell-to-cell communication.[5] The circular pads (20 μm diameter) were designed to adhere a cell body to each of these sites. Both circuit patterns (referred to as RT6 and RT12) had a 7 μm track width, but the overall feature size (length x width) was 520 μm x 270 μm for RT6 and 160 μm x 460 μm for RT12 (see Figure 7).

Photolithographic Patterning Process. An Ar/F excimer (λ = 193 nm) laser (Lambda LPX210), customized with a beam homogenizer (Exitech, TecOptics, Merrick, NY), was used for all experiments. The beam homogenizer has a lens array of 36 elements, designed to achieve a homogeneity of ± 5%. The laser was used in the constant hv mode (18.4 kV) with a repetition rate in the 20-30 Hz range. An 8 J/cm^2 laser dose was required for the circuit pattern design with a

beam area of 2.5 cm^2. For the circuit model, the energy per unit area was 3.0 ± 0.2 mJ/cm^2-shot (as measured by an Astral AA30 power/energy meter) with a burst count of 3000 ± 100 shots, resulting in a pulse time of approximately 2 min. For the line-space pattern, a 10 J/cm^2 laser dose with a burst count of 3700 ± 100 shots resulted in an approximately 3 min. pulse time (at the same energy and beam area as reported for the circuit pattern).

For pattern fabrication (Figure 1), a DETA-modified coverslip was irradiated through a photolithographic mask as described above. The UV light served to remove the cytophilic DETA in the exposed areas, creating a reactive surface which was then backfilled with the cytophobic silane 15F.

XPS Characterization. VG Scientific XPS Model ESCALAB 220i-XL is capable of both real-time imaging and high-energy resolution spectroscopy. The XPS was used in both modes for this work. Chemical imaging via XPS has been previously reported.[28,29] In the high-energy resolution mode, the takeoff angle was 35° and the spectra were normalized to the Si 2p peak of the substrate. To image patterned DETA the surface was first modified with a Pd catalyst as described by Dressick *et al.*[32,33] for selective electroless Ni deposition. In this manner, we can increase the signal from the nitrogen (N 1s) signal because the Pd complexes to the amine groups of the DETA. If it was desired, we could further react the Pd with a Ni bath to produce both an optical and a chemical image of the pattern. With the VG instrument, spatial resolution of a few microns is attainable for our patterned surfaces.

Contact Angle Measurements. Using the sessile drop technique,[34-36] a drop of deionized water was dispensed at the silanated surface via the microsyringe of an NRI Contact Angle Goniometer Model 100-00 (Ramé-Hart, Inc.) to measure the surface wettability. Both the advancing and receding contact angles were measured for each surface.

Cell Culture Conditions. Hippocampal neurons were isolated from E18-E19 Sprague-Dawley rat (Taconic Farms, Germantown, NY) embryos by papain dissociation (2 units/mL, Worthington Biochemical Corp., Lakewood, NJ). After dissociation, the cells were centrifuged, resuspended, layered over a step gradient, centrifuged through the gradient to remove debri, and then resuspended in the culturing media.[37] The cells were counted using a hemacytometer and plated at a density of 56×10^4 cells per 22 mm x 22 mm coverslip. The cells were cultured in a serum-free Neurobasal medium (Life Technologies, Gibco BRL, Grand Island, NY) supplemented with B27 (Life Technologies, Gibco BRL, Grand Island, NY), glutamine (0.5 mM, Life Technologies, Gibco BRL, Grand Island, NY), and glutamate (25μM, >99% TLC, Sigma Chemical, St. Louis, MO)[38] at 37°C and 5% CO_2.[39] At Days 2, 4, 8, (and subsequent multiples of 4) *in vitro*, 50% of the media was removed and replaced with Neurobasal medium supplemented with B-27 and glutamine (*i.e.*, without the glutamate). The removal of the glutamate was necessary to prevent neurotoxicity.[38]

We report statistical averages for pattern fidelity. As the mask allows for 100 circuit R16 patterns to be created per coverslip, cell pattern fidelity was tabulated as ± out-of-100 positive circuit patterns per coverslip. The number of cells attached at the circular somal sites were tabulated and averaged. We used an inverted microscope (Olympus CK2, Opelco, Sterling, VA) adapted with a digital CCD camera (3CCD, Dage-MTI, Inc, Michigan City, IN) and an Intel Pentium notebook computer (Travel Mate, Texas Instruments) to acquire and store pattern images, and Image-Pro Plus software (Cybernetics) to tabulate the average number of neurites per cell and the average neurite length.

Immunocytochemistry Procedure. The cells were preserved in 4% para-formaldehyde in Dulbecco's phosphate buffered saline (D-PBS). After the cells were exposed for 30 minutes to the para-formaldehyde fixative, they were rinsed with D-PBS (Quality Biologicals, Inc.) and refrigerated. For immunostaining, the fixed plates were incubated with mouse monoclonal anti-MAP-2 (1:300, Sigma) overnight at 4°C. After a D-PBS rinse, the cells were incubated in FITC-conjugated donkey anti-mouse IgG (1:50, Jackson Immunological Research) for 45 minutes at room temperature. The labeled cells were examined on an epifluorescence microscope (Zeiss) with a water immersion 25X objective.

Electrophysiology Experiments. Whole cell patch clamp techniques were carried out on pairs of patterned neurons to record functional connections. Dual patch clamp recordings were carried out using current- and/or voltage-clamp configurations. We looked for evidence of synaptic-like transient signals that

were either **spontaneous** occurrences or **evoked** responses. Spontaneous occurrences were the natural electrical activity of cells during development that appear random in nature. In the case of evoked responses, a voltage was applied to one (pre-synaptic) cell and a current signal from the second (post-synaptic) cell is recorded (Figure 2). The electrophysiology setup consisted of a L/M EPC-7 Patch Clamp System (LIST-Medical), an inverted microscope (Zeiss), a Model 7313 oscilloscope (Tektronix, Inc.) with simultaneous readout from both a CRC VR-100B Digital Recorder (INSTRUTECH Corp.) and a Brush 260 chart recorder (Gould). The cells were sealed with glass patch pipettes filled with a buffered intracellular Ca^{2+}-ATP solution. The extracellular solution was Tyrode's solution.[3] Membrane potentials were consistently held at -80mV.

Results

Our report on the fabrication of the cell patterns proceeds through several steps from chemistry and photolithography to cell culture and finally electrophysiological characterization of cell function.

A. Characterization of Unpatterned Aminosilane Surface.

It has been reported previously[40] that DETA promotes the adhesion and growth of hippocampal neurons. Cell culture on an unpatterned cytophilic DETA surface resulted in hippocampal neurons which were phase dark, indicating strong adherence, with extended neurites (Figure 3a). High resolution XPS for the DETA surface preparation yielded a nitrogen (N 1s) intensity of 3600

± 900 counts when normalized to a silicon (Si 2p) peak area of 5000. This new DETA surface preparation shows increased surface coverage and stability as compared to the previously reported methanol DETA surface preparation, which yielded a nitrogen (N 1s) intensity of 1300 ± 300 counts when normalized to a Si 2p peak area of 5000.[23] The advancing and receding contact angles for the new DETA preparation with their standard deviations were θ_{adv} = 42° ± 3° and θ_{rec} = 6° ± 5°, respectively.

B. Characterization of Unpatterned Fluorinated Silane Surface.

In contrast to DETA, 15F (much like the previously reported 13F)[23] impedes neuronal adhesion and neurite outgrowth. The hippocampal neurons become phase bright, indicating poor adhesion to the surface, with the cell bodies appearing clumped and the neurites fasiculated (Figure 3b). High resolution XPS for the 15F surface preparation yields a fluorine (F 1s) intensity of 14,200 ± 400 counts when normalized to a Si 2p peak of 5000. This compares to our previous methanol 13F surface preparation, which yielded a fluorine (F 1s) intensity of 8230 ± 3000 counts when normalized to a Si 2p peak of 5000.[23] The advancing and receding contact angles for 15F with their standard deviations were θ_{adv} = 90° ± 1° and θ_{rec} = 79° ± 5°.

C. Fabrication and Analysis of Circuit Patterns by XPS.

We have previously used deep UV laser patterning to reproducibly create line-space patterns.[23] However, when we moved to the more complex, higher resolution pattern designs, we found that we were unable to routinely produce

high quality patterns as evidenced by the optical images provided from Pd/Ni metallization and poor cell fidelity to the patterns. The non-uniform energy density distribution of the laser beam at the substrate surface[21] is evidenced in the optical photo shown in Figure 4a. We therefore utilized a beam homogenizer to deliver a top-hat energy profile of the laser beam at the substrate surface, resulting in uniform patterning as shown in the optical photo in Figure 4b. Patterns fabricated in this way yielded more uniform images in XPS and greater fidelity of cells to the patterns.

After incorporation of the beam homogenizer the best laser dosage for the circuit pattern design was determined. An optimal laser dose must be high enough to remove the aminosilane (DETA) during the patterning process, but low enough to prevent narrowing of the line widths and ablation of the right-angled features of the circuit design as seen in Figure 5. XPS analysis showed that a 5 J/cm^2 laser dose was necessary to remove 50% of the amine (N1s signal) from the surface and a 15 J/cm^2 dose was required to clear the amine from the surface. The best circuit patterns were found with a dose of 8 J/cm^2, as shown by imaging XPS in Figure 6.

D. Characterization of Cells Adhered to Circuits.

(1) Cell Adhesion to Individual Circuit Patterns. The ability to control surface composition coupled with the appropriate media conditions has shown to be effective in influencing neuronal development *in vitro*.[22] In our experiments, the E18-19 hippocampal neurons were plated in the B-27-

supplemented medium and circuit patterns were observed as early as one hour after cell plating. Staining with MAP-2 at Days 2 and 7 confirmed that the cells were neuronal. Both the circuit (Figure 7) and line-space patterns (Figure 8a) were successful in controlling the cell adhesion and neurite outgrowth. Cell fidelity was monitored to the optimized RT6 circuits (n = 400) with their somal adhesion sites (n = 800) at each time interval *in vitro*. Two hours after plating, 23% ± 12% fidelity to individual patterns and 17% ± 6% somal site fidelity was observed. Cell pattern fidelity peaked on Day 1 with 75% ± 15% pattern fidelity and 30% + 5% somal fidelity. On Day 2, the pattern and somal fidelity were 68% − 6% and 26% ± 2% respectively. Although the cells continued to be viable, the pattern fidelity declined through Day 7, when 1% pattern fidelity remained. Cell pattern fidelity for the line-space patterns was >90% on Day 1 and declined through Day 14.

(2) Increased Variance in Cell Morphology on Circuits. The cell morphology showed differences between patterned and unpatterned cells at the early time points. On Days 1 and 2, the cells on the unpatterned DETA surfaces were phase dark with extensive growth cones with short intertwined neurites mostly confined to the growth cone area (Figure 8b). At the same time points, the cells on the patterned surfaces (Figures 7 and 8a) lacked the extensive growth cones, but a greater variance in neurite length was observed. The average neurite length and number of primary neurites per cell on the RT6 circuits on Day 2 was 59.3 μm ± 35.3 μm and 2 + 1, respectively. For the line-space patterns,

the average neurite length and number of neurites per cell on Day 2 was 47.6 μm − 25.5 μm and 2 ± 1, respectively. For the unpatterned DETA-modified surfaces, the average neurite length and number of neurites per cell on Day 2 was 31.0 μm ± 14.3 μm and 2 ± 1, respectively.

(3) Electrophysiology of Circuits. The indication of synaptic activity is shown with neurotransmitters, which migrate across the synaptic cleft to the dendrites of the postsynaptic cell after release from axonal endings and are necessaryfor functional synaptic activity.[5] In the *adult* mammalian central nervous system, GABA is the major inhibitory neurotransmitter.[41-44] In immature rats, GABA depolarizes hippocampal neurons and increases intracellular [Ca^{2+}] and is generally considered excitatory.[45] The switch from excitatory to inhibitory comes early in postnatal development, but its presence is indicative of synaptic development. The other major neurotransmitter found in the adult mammalian cells is glutamate, which is excitatory. GABA is produced from glutamate in the presence of glutamate decarboxylase (GAD).[42] Recently, Dupuy and Houser[46] discovered that GAD-67 and GAD-65 containing neurons could be observed as early as E17-E18 in the rat hippocampus indicating the presence of both neurotransmitters in early embryonic development.

The dual patch clamp technique was used to look for both spontaneous and evoked synaptic activity over the time course of the hippocampal cell development on both the patterned and unpatterned silanated substrates. In the B27-supplemented Neurobasal serum-free defined medium,[38] the GABA

(gamma-aminobutyric acid)[11,45,47,48] initiated synaptic signals developed over time in culture. Recordings during the first 6 days after cell plating for **both** the unpatterned and patterned surfaces gave no evidence of either spontaneous or evoked synaptic activity *in vitro*. By Day 7 or 8, we recorded both spontaneous and uni-directional evoked synaptic signals for the **unpatterned** DETA surface (Figure 9a). However, we observed only spontaneous activity (i.e., a lack of evoked response) for the **patterned** (line-space and circuit patterns) surfaces at Day 7 or 8 (Figure 9b). By Day 12, the cells on the line-space patterned surface displayed **both** spontaneous and uni-directional evoked synaptic signals (Figure 10b). The Day 12 cells on the unpatterned DETA surface displayed both spontaneous activity and a preference for evoked signals in one direction (Figure 10a). The longevity of the circuit patterns was about a week, so we were unable to provide the Day 12 data for this particular pattern for comparison. However, it is interesting that the same trend in signal emergence is observed through the entire first week for the cells on both the line-space and circuit patterned surfaces.

Since adsorbed poly-D-lysine is generally considered to be a biological surface compatible for cell culture and differentiation,[37] in a complementary set of experiments, we observed parallel synaptic development of the hippocampal neurons on unpatterned DETA and unpatterned poly-D-lysine. The results reinforced previous cell-surface data[37] which assessed DETA as a suitable and comparable artificial surface for cell culture.

Discussion

Using patterned artificial surfaces we have achieved a high degree of neuronal fidelity to patterned circuits as determined by the percentage of cell circuit patterns formed on the high resolution DETA/15F templates. In a defined medium, the neurons were able to reproduce some fundamental developmental processes under the conditions of geometric constraint on the circuit patterns. The steps we have outlined here combined with appropriate surface analysis should now allow a large number of researchers access to this methodology.

The achievement of reproducible patterns has allowed us to begin monitoring the effects of patterns in statistically significant numbers. The lack of extensive growth cones for the patterned neurons and a greater variance in neurite length indicate that geometric constraint affected the cell morphology at early time points. Mennerick *et al.*[43] recently suggested that for postnatal rat hippocampal neurons that constrained microisland neurons elaborated less extensive neuritic arborizations than neurons cultured randomly. Collectively, these results imply that geometry is a contributing factor for earlier, directed growth of neurites.

In spite of the earlier accelerated growth for hippocampal neurons on patterned surfaces, we observed greater longevity on the unpatterned DETA surfaces. In fact, the order of hippocampal neuron longevity for the various surfaces was consistently as follows: DETA, unpatterned (longevity of 1 month) > DETA/15F, line-space pattern (longevity of 2 weeks) > DETA/15F, circuit

pattern (longevity of 1 week). For the patterned cells, the term longevity is meant to imply the length of pattern recognition, not cell viability, as the cells often continued to live long after the neurites had overgrown the pattern feature. We previously reported the use of XPS to monitor the deposition of protein in serum-free media at the artificial surface-cell interface.[21,37] We hypothesize in the present case that the protein deposition may be obscuring the patterns with time. We further hypothesize that the difference in the longevity of pattern recognition between the line-space and circuit patterns may be due to cell density factors as the pattern features get smaller the number of healthy neurons decreases as more cytophobic surface is present. Diffusable factors from the cells have been shown to affect longevity and the concentration of these factors decreases as cell density decreases, lowering survival times. We will investigate these potential factors with new mask designs for further optimization in future work.

The synaptic development is also of keen interest to our group and the patterns were used to explore this fundamental developmental process. *In vivo* the rat hippocampus is recognizable prenatally on embryonic (E) Day 16. The region is postulated to develop on E15 with the connection of two primordia in the telencephalon. The most rapid growth occurs between E16 and E17 with a volumetric increase of 1900%. The embryonic birth date is E22, but the volumetric expansion of the hippocampus continues through postnatal (PN) Day 21.[40] So in the present study we are starting with very immature hippocampal

neurons that have not established connections yet. This allows us to study synaptic development from the initial periods of hippocampal development.

In the present work, we used dual patch clamp electrophysiology to monitor the emergence of synaptic activity *in vitro* for hippocampal neurons dissociated with papain from E18-E19 rats. We wanted to determine if patterned artificial surfaces could influence the synaptic development *in vitro* and to possibly provide clues with regard to *in vivo* development. In all of the evoked synaptic activity (responses to electric pulses through the patch clamp), we observed physiological responses of GABAergic origin as determined by the electrophysiological signature. On the unpatterned DETA-modified surfaces we observed both spontaneous and evoked activity by Day 7 or 8. On the patterned surfaces (*i.e.*, line-space and circuit designs) we observed only spontaneous currents by Day 7 or 8 with the evoked activity being delayed until Day 12 for the patterned (line-space) surface. This delayed onset of evoked activity occurred despite the earlier accelerated growth and visible formation of overlapping connections for the patterned axons and dendrites. In addition, the evoked synapses always showed a uni-directional preference. Although a direct correlation between *in vitro* cultures and slices cannot yet be made, it is well established that in slice preparations glutamatergic synaptic activity is uni-directional from CA1 to CA3 neurons.[8] We conclude that the geometric control by patterning is a modulating or trophic[1] factor for both the cell morphology and

the neuronal synaptic development. Our goal now is to begin developing this new tool to *control* synaptic activity for *in vitro* model systems.

Conclusions

Using XPS, advances in surface preparation, optical microscopy, and a beam homogenizer for a more uniform deep UV photolithography, we have achieved uniform, routine high resolution photolithographic circuit patterns on artificial surfaces. We have achieved a >50% rate of pattern formation and at times the rate approached 90% for the optimized high resolution circuit patterns using specific media conditions. With the defined neuronal connections created by the patterns, we were able to begin exploring the fundamental issue of how geometry affects both cell morphology and synaptic development *in vitro*. We conclude that geometry itself may be a modulating or trophic factor allowing for greater variance in neurite outgrowth at early time points, and delaying synaptic development for the patterned cells until later time points. We are trying to relate this work to processes that occur *in vivo* during development to provide a reproducible model system for studying *in vivo* development *in vitro*.

We plan to use our high fidelity, high resolution circuit patterns to study many aspects of neuronal development in a controlled manner. New mask designs will now be used to control the parameters in our *in vitro* system and be used to investigate how varying geometry affects neuronal development.

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Figure Captions:

Figure 1. For pattern fabrication, a cytophilic DETA-modified coverslip is irradiated with 193-nm laser light through a photolithographic mask. The resulting reactive surface is then chemically backfilled with the cytophobic silanated 15F.

R = $(CH_2)_2$-NH-$(CH_2)_2$-NH-$(CH_2)_2$-NH_2 and R' = Si-$(CH_3)_2$-$(CH_2)_2$-$(CF_2)_7$-CF_3

Figure 2. Schematic illustration of the experimental arrangement for dual patch clamp recording. Both neurons are voltage-clamped and the current signal is recorded for both spontaneous (single neuron) occurrences and evoked (pre-synaptic neuron to post-synaptic neuron) responses.

Figure 3. Day 4 *in vitro* hippocampal neurons at 20X magnification plated onto unpatterned (a) DETA-coated glass coverslip where the cells appear phase dark and (b) 15F-coated glass coverslip where the cells appear phase bright with fasiculated (bundled) neurites.

Figure 4. Optical images of Pd/Ni metallized RT6 circuit images prepared (a) without the beam homogenizer and (b) with the beam homogenizer.

Figure 5. Optical images (20X) of Pd/Ni metallized RT6 circuit at laser dosages of (a) 5 J/cm^2, (b) 8 J/cm^2, (c) 10 J/cm^2, and (d) 15 J/cm^2.

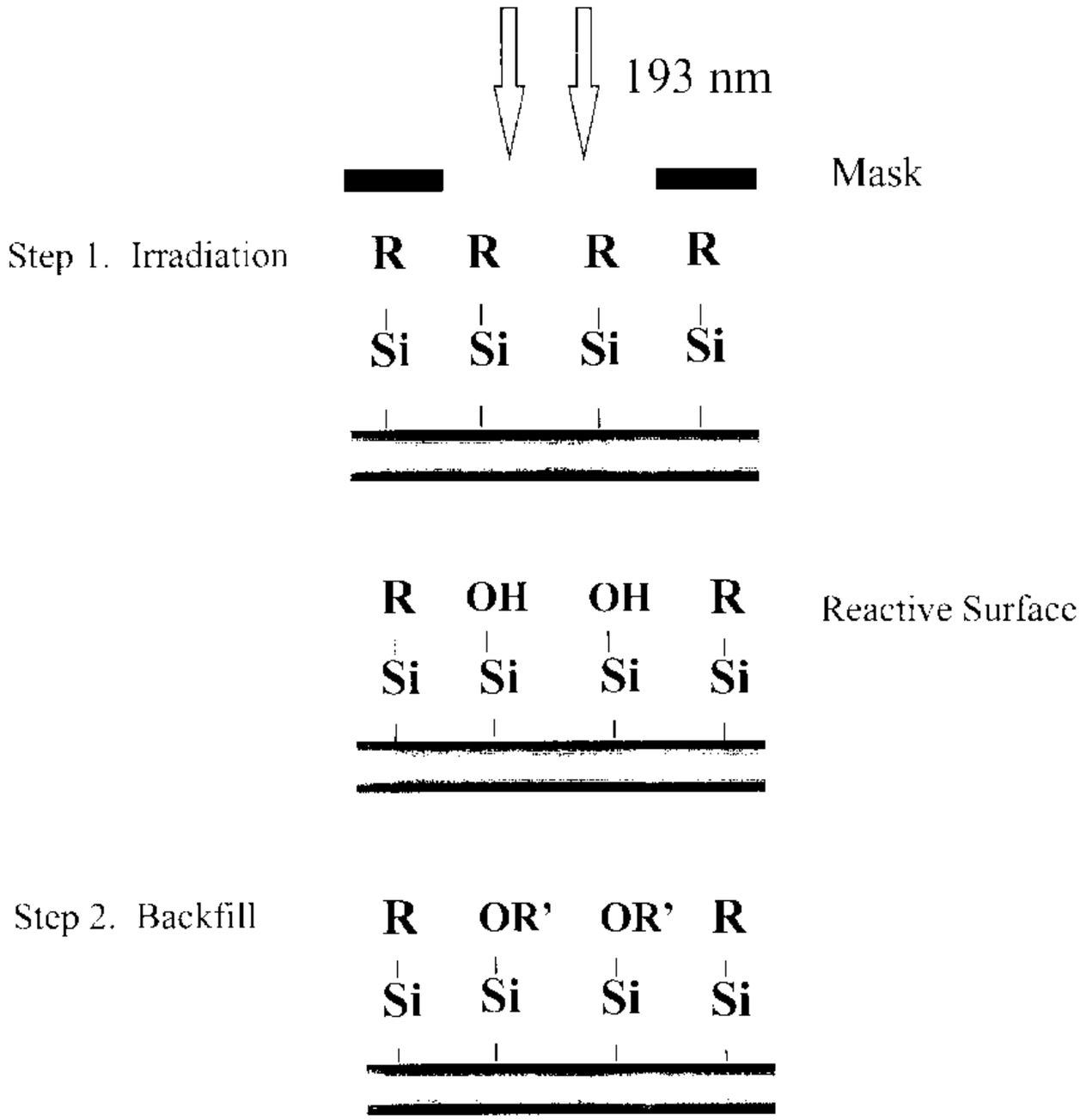
Figure 6. XPS images of (a) a circuit RT6 pattern and (b) a circular somal adhesion site. In both cases, the patterns are metallized with Pd/Ni and the Ni 3p signal is imaged.

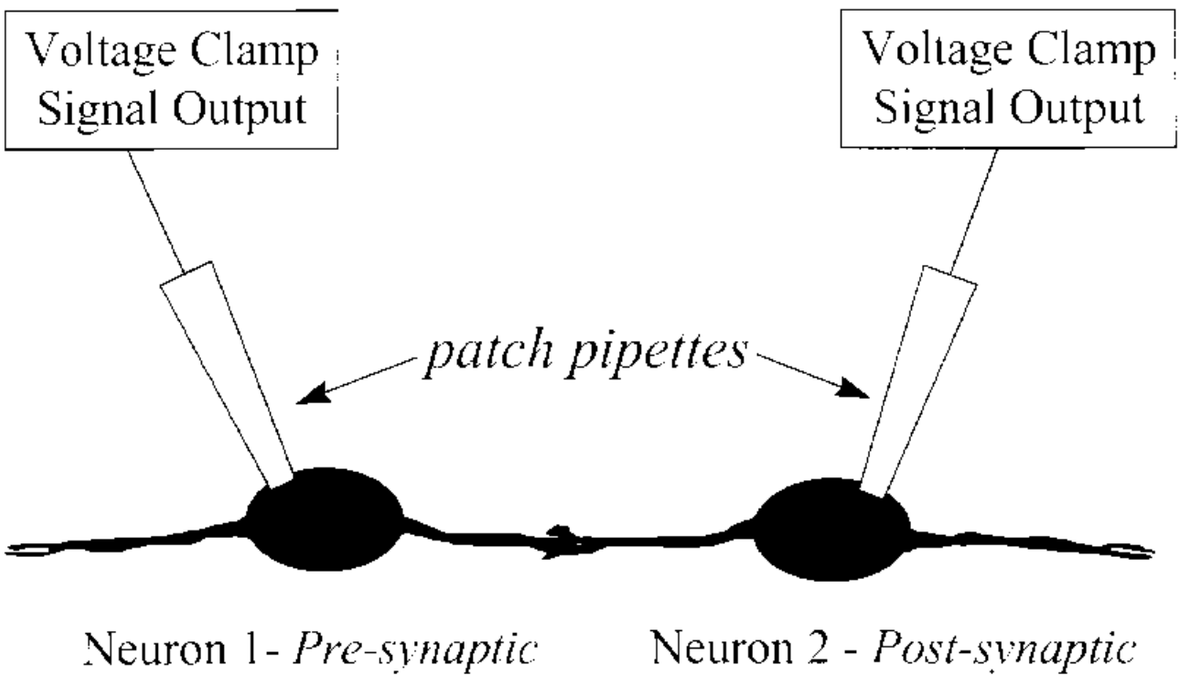
Figure 7. Photographs (20X) of the (a) circuit RT12 mask feature and the patterned Day 2 *in vitro* hippocampal neurons; (b) circuit RT6 mask feature and the patterned Day 2 *in vitro* hippocampal neurons.

Figure 8. Day 2 *in vitro* hippocampal neurons (20X) plated onto (a) a DETA/15F line-space pattern and (b) unpatterned DETA.

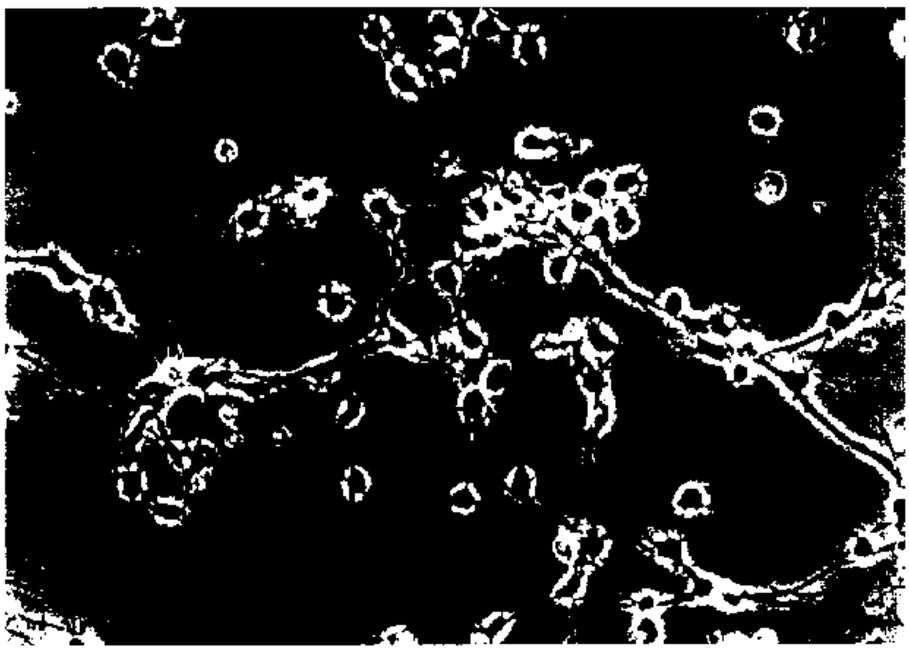
Figure 9. Electrophysiology of Day 8 *in vitro* hippocampal neurons on (a) an unpatterned DETA-modified surface displaying both spontaneous and evoked activity, and (b) a DETA/15F circuit patterned surface displaying only spontaneous activity. In both cases, Neuron 1 is pre-synaptic and Neuron 2 is post-synaptic.

Figure 10. Electrophysiology of Day 12 *in vitro* hippocampal neurons displaying both spontaneous and evoked activity on (a) an unpatterned DETA-modified surface and (b) a DETA/15F line-space patterned surface. In both cases, Neuron 1 is pre-synaptic and Neuron 2 is post-synaptic.

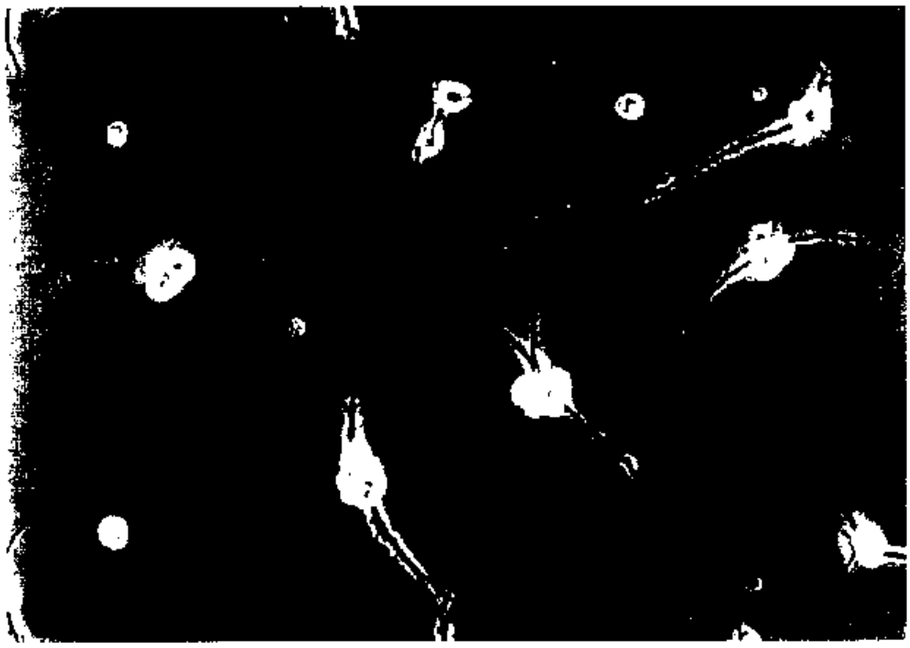

193 nm
Mask
Step 1. Irradiation
R R R R
Si Si Si Si
R OH OH R
Si Si Si Si
Reactive Surface
Step 2. Backfill
R OR' OR' R
Si Si Si Si


Voltage Clamp
Signal Output
Voltage Clamp
Signal Output
patch pipettes
Neuron 1 - Pre-synaptic
Neuron 2 - Post-synaptic

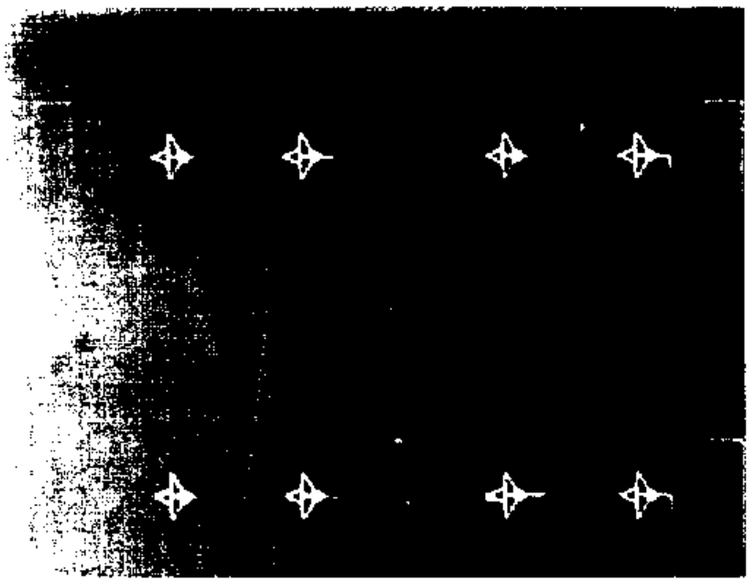
(a)



(b)



(a)



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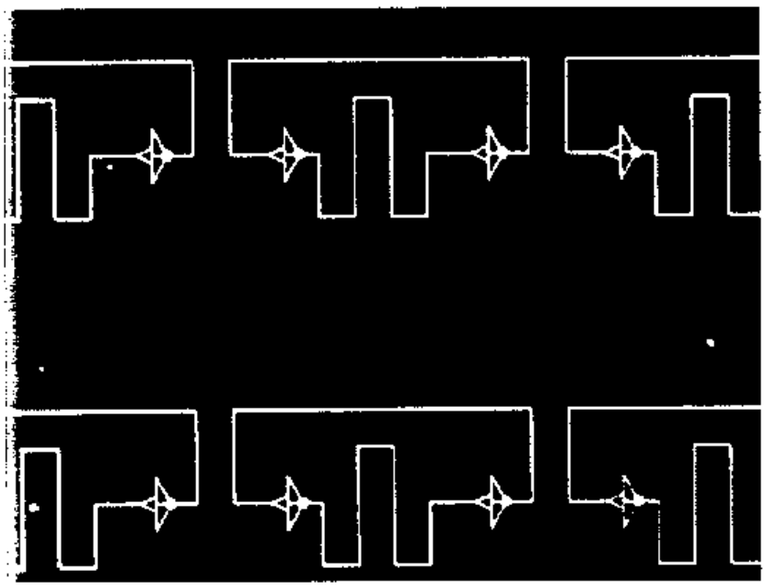

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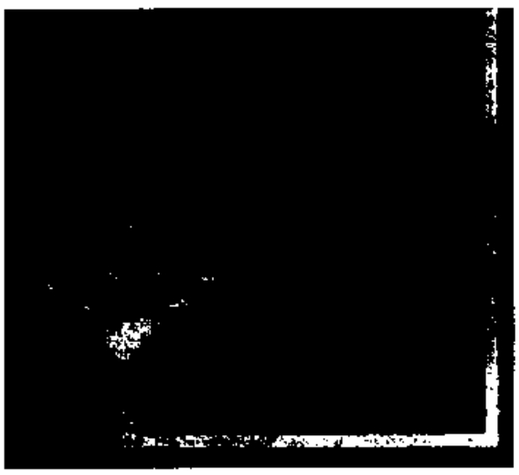

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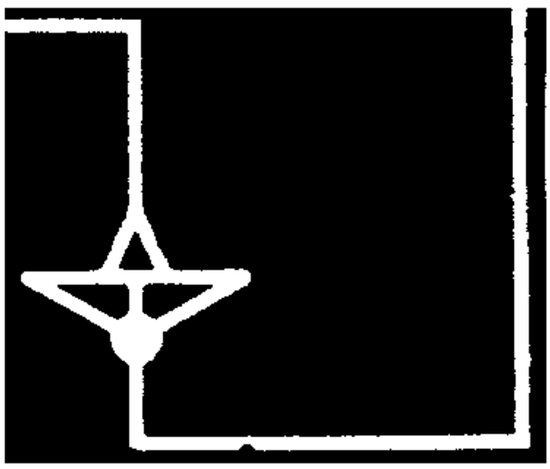
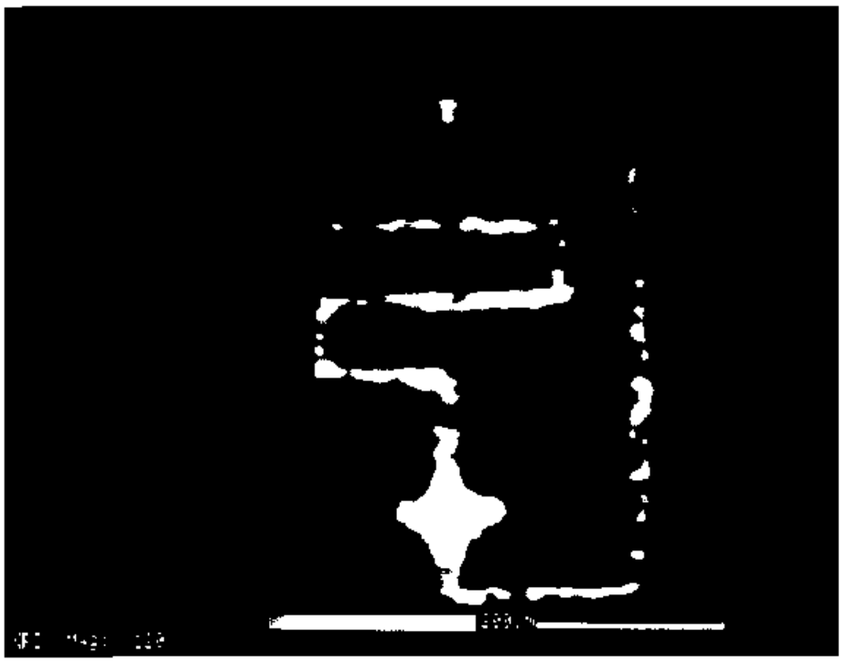


(c)

(d)

(a)



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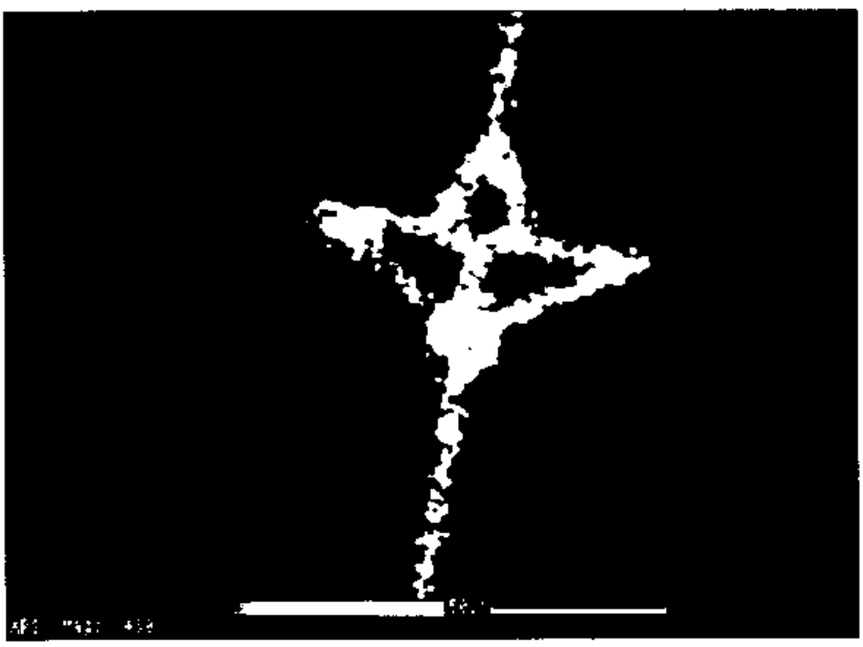
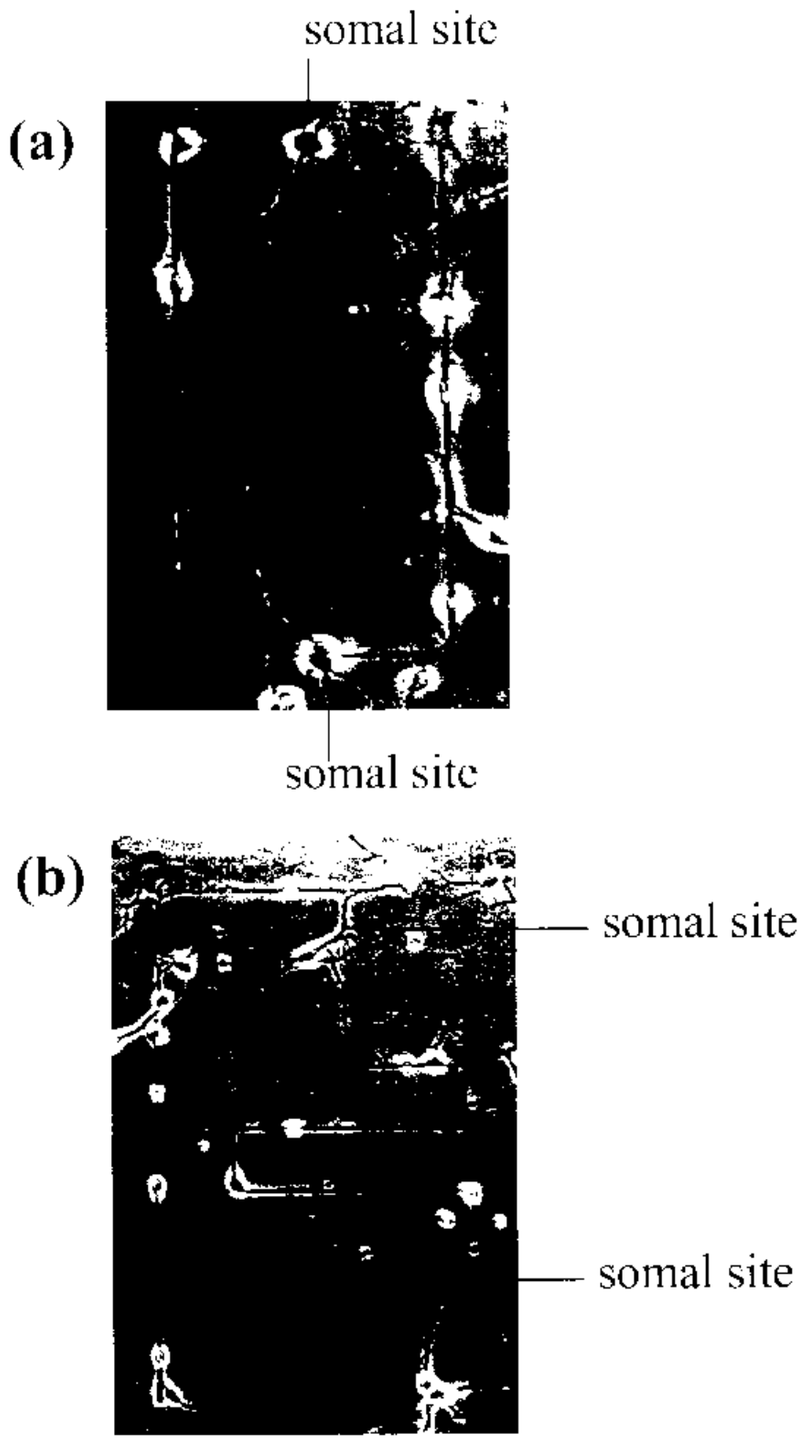
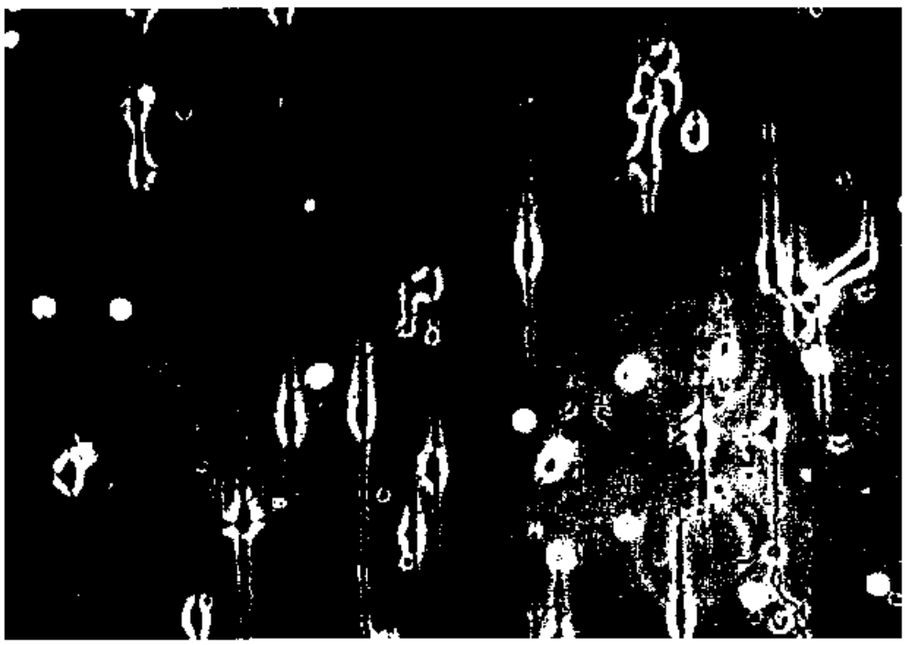



somal site
(a)
somal site
(b)
somal site
somal site

(a)



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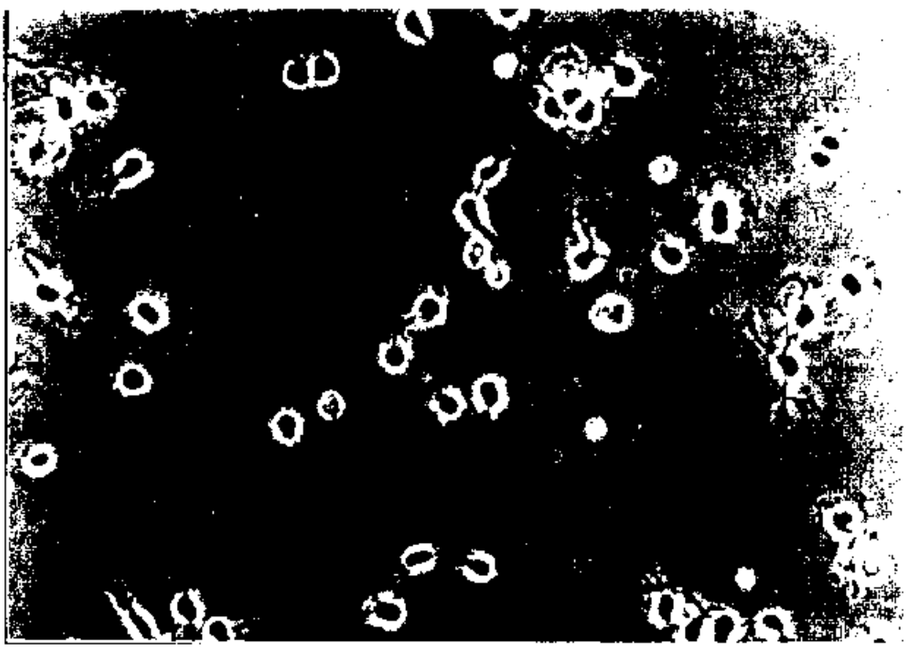

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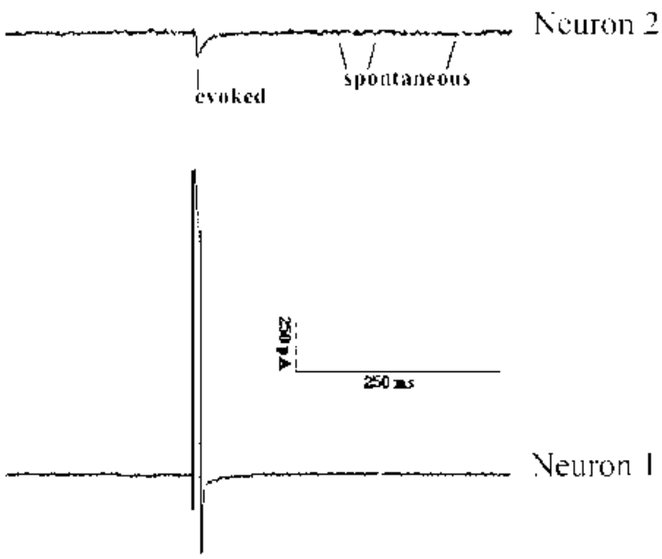
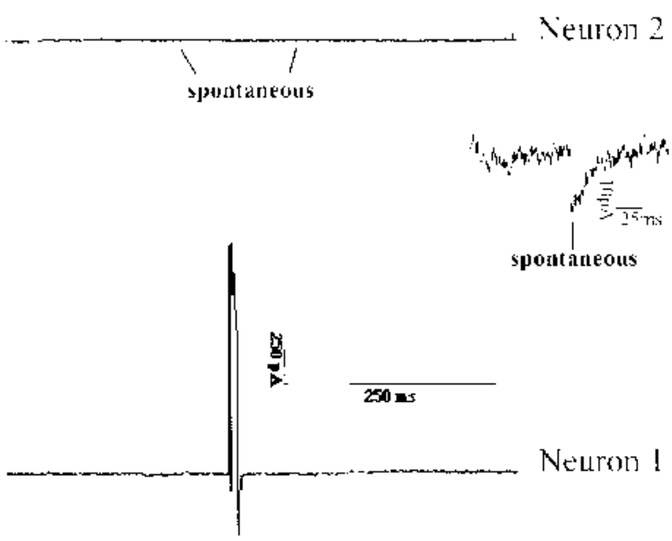
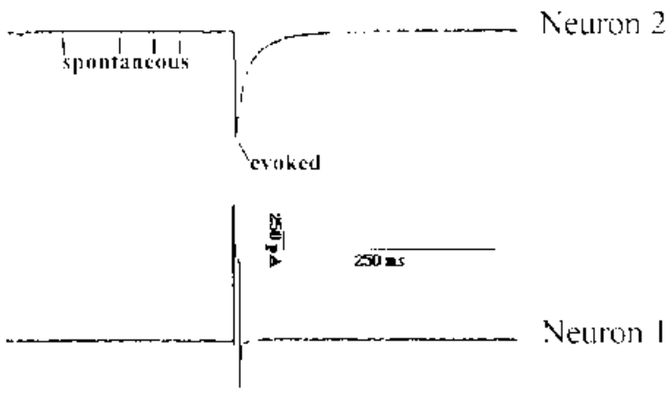

Neuron 2
evoked
spontaneous
250 pA
250 ms
Neuron 1

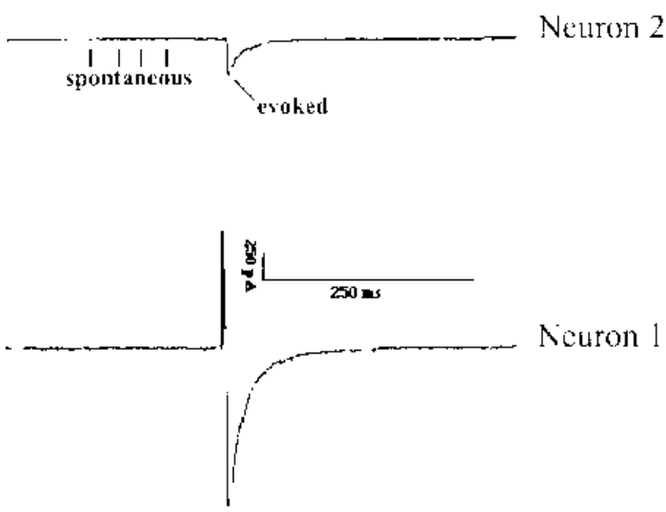
(b)


Neuron 2
spontaneous
10 pA
25 ms
spontaneous
250 pA
250 ms
Neuron 1

(a)


Neuron 2
spontaneous
evoked
250 pA
250 ms
Neuron 1

(b)


Neuron 2
spontaneous
evoked
250 pA
250 ms
Neuron 1